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Correlation of MDV vaccine virus genome load in feather tips with protection, using an experimental challenge model

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Susceptibility and transmissibility of pigeons to Asian lineage highly pathogenic avian influenza virus subtype H5N1

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Abstract

To determine whether or not pigeons are susceptible to infection with Asian lineage highly pathogenic (HP) avian influenza virus (AIV) subtype H5N1 and can serve as a transmission host for H5N1 HPAIV, we experimentally infected 187 young and adult pigeons with five different isolates of H5N1 HPAIV and co-habited some experimentally-infected pigeons with susceptible specific pathogen free (SPF) chickens. Results showed that all infected pigeons remained clinically healthy during the observation period. No gross lesions or histopathological changes were observed in the infected pigeons, and haemagglutination inhibition (HI) antibodies were not detected in serum samples of the infected pigeons. Additionally, all chickens placed in contact with AIV H5N1 infected pigeons remained healthy, and no virus or HI antibodies were detected in samples from the chickens. Our data suggest that pigeons are not susceptible to Asian lineage HPAIV H5N1 and do not transmit the virus to chickens.

Introduction

Asian lineage highly pathogenic (HP) avian influenza virus (AIV) of subtype H5N1 first emerged in 1996 and has caused significant economic losses to the poultry industry (Li *et al.*, 2004; Xu *et al.*, 1999). Since the end of 2003, the Asian lineage H5N1HPAIV spread rapidly across eastern Asia and raises great concern for the emergence of human influenza A pandemic virus infection due to the parallel human H5N1 influenza cases in some countries (Chan, 2002; Li *et al.*, 2004; Shinya *et al.*, 2006). A wide range of wild and migratory birds, waterfowl, and wild mammals, including tigers and cats, are susceptible to Asian lineage AIV H5N1 infection (Keawcharoen *et al.*, 2004; Liu *et al.*, 2005; Perkins & Swayne, 2002a, 2002b, 2003). Although Asian lineage H5N1 HPAIV has been isolated from pigeons in field samples (Ellis *et al.*, 2004; Li *et al.*, 2004), the existing evidence indicates that pigeons are only minimally susceptible or resistant to different subtypes of AIV infections (Fang *et al.*, 2006; Kaleta & Honicke, 2004; Lillehaug *et al.*, 2005; Panigrahy *et al.*, 1996; Perkins & Swayne, 2002). One study demonstrated that even immune-suppressed pigeons were resistant to low pathogenic AIV subtypes (Fang *et al.*, 2006). Other studies suggested that pigeons were more susceptible to some H7 strains, than to the H5 strains tested, but that they might serve as a mechanical transmission host for AIV (Kaleta & Hönicke, 2004). Similarly, pigeons were found to be resistant to infection with A/chicken/Hong Kong/220/97 H5N1 HPAIV (Perkins & Swayne, 2002). Even when pigeons received a high dose (10^8 EID₅₀) of A/chicken/Indonesia/2003 H5N1 HPAIV experimentally, only about 21% of the pigeons exhibited neurological distress and 65% of the pigeons and all sentinel chickens remained

healthy (Klopfleisch *et al.*, 2006). Therefore, the potential role of pigeons in establishing infection and spreading Asian lineage H5N1 HPAIV remains controversial. A number of factors including age and species of pigeons, genetic variations of different H5N1 HPAIV isolates, and route and dosage of virus inoculation may be important in determining the susceptibility of pigeons to AIV. The purposes of the present study were to examine in greater detail whether different types of pigeons, young or adult, are susceptible to different isolates of Asian lineage H5N1 HPAIV by using different routes of inoculation, and to determine whether experimentally infected pigeons can transmit the virus to susceptible chickens when placed in contact with them.

Materials and Methods

Animals. One hundred and eighty-seven healthy squabs and mature pigeons, including both male and female racing, town and meat types, were obtained from the Beijing Conference Center. All animals were negative for haemagglutination inhibition (HI) antibodies to the H5 subtype of AIV. Forty-nine 5- to 16-week-old specific pathogen free (SPF) chickens and 10-day-old embryonated SPF fowls eggs were provided by the Beijing Experimental Animal Research Center (Beijing, China). Animals were housed in negative-pressure isolators. Animal protocols in this study were approved by the Beijing Animal Welfare Committee (SYXK, Beijing, 2002- 0010).

Viruses. A total of five H5N1 HPAIV isolates (A/Chicken/Huabei/H5N1/2004, A/Duck/Huanan/H5N1/2004, A/Goose/Huanan/H5N1/2004, A/Chicken/Huabei/H5N1/2006/01, and A/Chicken/Huabei/H5N1/2006/02) was used in this study. The viruses were isolated from chickens, ducks and geese, respectively, and from different regions in China during the outbreaks from 2004 to 2006. The intravenous pathogenicity index (IVPI) for each of the five isolates was determined as described (Slemons *et al.*, 1991). The IVPI value for each of the five isolates was 3.0, indicating that they were HPAIV. Each virus was propagated in 10-day-old embryonated SPF fowls' eggs, allantoic fluids were harvested aseptically and stored at -70°C . The median egg infectious dose (EID_{50}) of each isolate was determined by the method of Reed & Muench (Reed & Muench, 1938) before use.

Animal inoculation and experimental design. A total of 187 pigeons and 49 SPF chickens was used for the study. Six experiments were performed to investigate the susceptibility of young and adult pigeons to different isolates of Asian lineage AIV H5N1 via different routes of inoculation and to determine the transmissibility of AIV H5N1 from pigeons to susceptible in contact chickens. The protocols of the six experiments are summarised in Table 1. In all experiments, each pigeon and chicken was inoculated with 5×10^4 EID₅₀ of the Asian lineage H5N1 HPAIV isolates in 0.5ml. Clinical signs including depression, respiratory distress, diarrhoea and death were observed and recorded daily post infection. At defined days post inoculation, pigeons or chickens were selected randomly, tracheal and cloacal swab samples were collected for virus isolation, and serum samples were tested for H5 antibodies as described below. Necropsies were then performed to examine gross lesions and histopathological changes. The internal organs including liver, lungs, intestines, kidneys, heart, brain and spleen were collected for virus isolation.

Virus isolation. The internal organs were homogenized in 5ml of ice-cold PBS(20% w/v). Swabs and tissue homogenates containing heart, liver, spleen, kidneys, brain and lungs were filtered through a 0.22µm filter and used to inoculate five 10-day-old SPF fowls' eggs. Virus was then detected by haemagglutination (HA) tests on harvested allantoic fluids as described (Yamada *et al.*, 1985). All experiments were performed in the biosafety level 3 containment facilities at Beijing Municipal Academy of Agriculture and Forestry Science.

Histopathological examination. Heart, liver, spleen, kidneys, lungs, and intestine tissues

were collected, fixed in 10% phosphate-buffered neutral formaldehyde, and processed for paraffin embedding and routine haematoxylin and eosin (H.E.) staining.

HA and HI tests. Haemagglutination (HA) and haemagglutination inhibition (HI) tests were performed as described (Klopfleisch *et al.*, 2006) using the reference H5 antigen and H5N1 positive control serum provided by the Animal Disease Diagnosis and Control Lab of Institute of Animal Husbandry and Veterinary Medicine (Beijing, China).

Results and Discussion

Whether Asian lineage H5N1 HPAIV isolates are able to establish infections in pigeons and transmit the virus to other susceptible hosts is a controversial issue, but is an important concern for controlling HPAI. In this study, 187 young and adult pigeons were infected experimentally with five different isolates of Asian lineage H5N1 HPAIV by either intranasal (IN), intraocular and oral route (IO), intramuscular (IM), or intravenous (IV) routes (Table 1). Of the 187 inoculated pigeons, none of them exhibited any clinical sign of illness and all remained healthy during the observation period (14-21 days post infection). In contrast, all the chickens inoculated with the Asian lineage H5N1 HPAIV isolates died within 96 h after infection. All the pigeons examined showed no gross lesions, except one with a swollen spleen. In contrast all the chickens exhibited typical degeneration and necrosis of liver, focal necrosis of pancreas, deposition of urates in kidney tubules, haemorrhagic spots and ecchymosis in the myocardium, epicardial fat, proventriculus, and the gizzard wall. Virus was isolated initially from 2 of the 187 inoculated pigeons, while virus was isolated from all the chickens infected experimentally (Table 2). In addition, the samples from all pigeons were negative during the subsequent passages of virus isolates in SPF chicken embryos, suggesting that the two positive samples might have been due to residual virus inoculum in the inoculated pigeons. All pigeons were negative in HI tests on serum samples taken during the observation period. For the chickens that were housed together in the isolators with the infected pigeons, no clinical signs or gross lesions were seen, and no virus or HI antibodies were detected, indicating that pigeons were not only resistant to AIV H5N1 infection, but also did not shed virus and transmit the virus to susceptible chickens (Table 2). In contrast, seven

of the eight SPF chickens in contact with chickens infected experimentally developed HPAIV clinical signs and died on day 6 post co-habitation (Table 2). Overall, our results clearly indicate that both young and adult pigeons are resistant to the five highly pathogenic Asian lineage H5N1 HPAIV isolates regardless of the route of inoculation, and pigeons do not serve as a transmission host of the virus.

Previous studies have analyzed the susceptibility of pigeons to AIV strains showing high and low pathogenicity for chickens. Pigeons were resistant or minimally susceptible to both high pathogenic AIV H5N2 and H7N7 and low pathogenicity H5N2 and H7N1 AIV (Panigrahy *et al.*, 1996). A recent review by Kaleta and Honicke concluded that pigeons are only partially susceptible to some H7 AIV strains and even less susceptible to the H5 AIV strains that had been tested (Kaleta & Hönicke, 2004). A recent study indicated that immune-suppressed pigeons were resistant to the low pathogenicity AIV subtypes H5N2 and H6N1 infections and did not transmit virus to susceptible hosts (Fang *et al.*, 2006). The results obtained from the present study are consistent with the above observations showing the resistance of pigeons to Asian lineage H5N1HPAIV. However, a report suggested that experimental infection with A/chicken/Indonesia/2003 (H5N1) via intraocular and intranasal administration routes could cause high morbidity and mortality in pigeons (Klopfleisch *et al.*, 2006). The conflicting results between our study and that of Klopfleisch *et al.*, may be partly due to the differences in viral dosage and virus isolates used in infection. In the study by Klopfleisch *et al.*, a dose of 10^8 EID₅₀ of the Indonesia/2003 H5N1 HPAIV was used (Klopfleisch *et al.*, 2006). We used 5×10^4 EID₅₀ of the five Asian lineage H5N1 HPAIV isolates from field outbreaks in China. Although, the role of pigeons as a natural transmission

host is still questionable, we believe the dose used in our study is more likely to be close to that seen in natural infections. Even when the higher dose of AIV was used, all sentinel chickens and approximately 65% of the inoculated pigeons remained healthy (Klopfleisch *et al.*, 2006). Therefore, based on the previous data and our results, it is reasonable to conclude that pigeons are relatively resistant to Asian lineage AIV H5N1 and may not serve as an efficient transmission host for the spread of the virus during natural infection. Further investigations on the molecular mechanism of the resistance of pigeons to the Asian lineage H5N1 HPAIV isolates described in the present study are needed.

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Table 1. *Summary of animal experiments*

Expt.#	Animals	Virus	Route of inoculation	Purpose of expt.
#1	13 mature pigeons	A/Chicken/Huabei/H5N1/2004	IN ^a , IO ^b	susceptibility
	14 mature pigeons	A/Chicken/Huabei/H5N1/2004	IM ^c	susceptibility
	5 SPF chickens	A/Chicken/Huabei/H5N1/2004	3 IM and IN and 2 IO	positive control
#2	15 squabs	A/Chicken/Huabei/H5N1/2004	IN, IO	susceptibility
	15 squabs	A/Chicken/Huabei/H5N1/2004	IM	susceptibility
	12 squabs	none	none	negative control
	4 SPF chickens	A/Chicken/Huabei/H5N1/2004	2 IM and 2 IN, IO	positive control
#3	16 squabs	A/Duck/Huanan/H5N1/2004	IN, IO	susceptibility
	16 squabs	A/Duck/Huanan/H5N1/2004	IM	susceptibility
	12 squabs	none	none	negative control
#4	16 squabs	A/Goose/Huanan/H5N1/2004	IN, IO	susceptibility
	16 squabs	A/Goose/Huanan/H5N1/2004	IM	susceptibility
	12 squabs	none	none	negative control
#5	15 25-50 day-old pigeons	A/Chicken/Huabei/H5N1/2006/01	IV ^d	transmissibility
	4 12-week-old SPF chickens	none	none	co-habiting ^e
	8 25-50-day-old pigeons	A/Chicken/Huabei/H5N1/2006/01	IM	transmissibility
	4 12-week-old SPF chickens	none	none	co-habiting
	4 12-week-old SPF chickens	A/Chicken/Huabei/H5N1/2006/01	IV, IM	transmissibility
	8 12-week-old SPF chickens	none	none	co-habiting
#6	7 25-50-day-old pigeons	A/Chicken/Huabei/H5N1/2006/02	IO	transmissibility
	4 12-week-old SPF chickens	none	none	co-habiting
	4-12-week-old SPF chickens	A/Chicken/Huabei/H5N1/2006/02	IO	transmissibility
	4 12-week-old SPF chickens	none	none	co-habiting

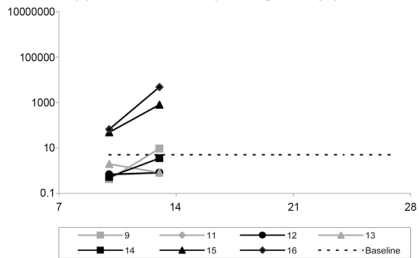
^a Intranasal; ^b Intraocular and oral; ^c Intramuscular; ^d Intravenous, ^e SPF chickens were placed in the same isolators with the experimentally infected pigeons or chickens.

Table 2. Summary of virus isolation results from animals

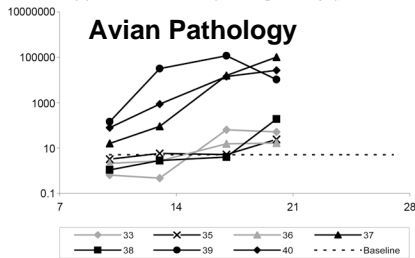
Expt.#	Animal groups	Virus isolation (positive/total birds) at defined days after inoculation										Passage	cumulative
		2d	4d	6d	7d	8d	9d	10d	14d	15d	21d		
#1	IN ^a +IO ^b pigeons	0/2	1/2	0/2	- ^c	0/2	-	-	0/2	-	0/3	Neg ^d	1/13
	IM ^c pigeons	0/2	0/2	1/2	-	0/2	-	-	0/2	-	0/4	Neg	1/14
	SPF chickens	5/5	-	-	-	-	-	-	-	-	-	NT ^f	5/5
#2	IN+IO pigeons	0/3	0/3	0/3	-	-	-	0/3	-	0/3	-	Neg	0/15
	IM pigeons	0/3	0/3	0/3	-	-	-	0/3	-	0/3	-	Neg	0/15
	SPF chickens	4/4	-	-	-	-	-	-	-	-	-	NT	4/4
	Control pigeons	0/2	0/2	0/2	-	-	-	0/3	-	0/3	-	Neg	0/12
#3	IN+IO pigeons	0/2	0/2	-	0/2	-	0/2	-	0/8	-	-	Neg	0/16
	IM pigeons	0/2	0/2	-	0/2	-	0/2	-	0/8	-	-	Neg	0/16
	SPF chickens	4/4	-	-	-	-	-	-	-	-	-	NT	4/4
	Control pigeons	0/2	0/2	-	0/3	-	0/2	-	0/3	-	-	Neg	0/12
#4	IN+IO pigeons	0/2	0/2	-	0/3	-	0/2	-	0/7	-	-	Neg	0/16
	IM pigeons	0/2	0/2	-	0/3	-	0/2	-	0/7	-	-	Neg	0/16
	SPF chickens	4/4	-	-	-	-	-	-	-	-	-	NT	4/4
	Control pigeons	0/2	0/2	-	0/2	-	0/2	-	0/4	-	-	NT	0/12
#5	IV ^g pigeons	0/2	-	0/2	-	-	-	0/2	-	-	0/9	NT	0/15
	SPF chickens with IV pigeons	-	-	-	-	-	-	-	-	-	0/4	NT	0/4
	IM pigeons	0/2	-	0/2	-	-	-	0/2	-	-	0/2	NT	0/8
	SPF chickens with IM pigeons	-	-	-	-	-	-	-	-	-	0/4	NT	0/4
	IM, IV chickens	4/4	-	-	-	-	-	-	-	-	-	NT	4/4
	SPF chicken with IM, IV chickens	-	1/1	6/6	-	-	-	-	-	-	-	0/1	NT
#6	IO pigeons	-	-	-	-	-	-	-	-	-	0/7	NT	0/7
	SPF chickens with IO pigeons	-	-	-	-	-	-	-	-	-	0/4	NT	0/4
	IO chickens	-	4/4	-	-	-	-	-	-	-	-	NT	4/4
	SPF chickens with IO chickens	-	-	4/4	-	-	-	-	-	-	-	NT	4/4

^a Intranasal; ^b Intraocular and oral; ^c No animals examined; ^d Negative; ^e Intramuscular; ^f Not tested; ^g Intravenous.

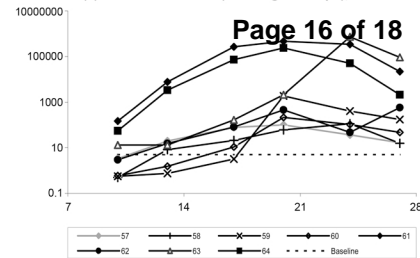
(a) 0.01 dose vaccine (challenged 14 dpv), Room 2



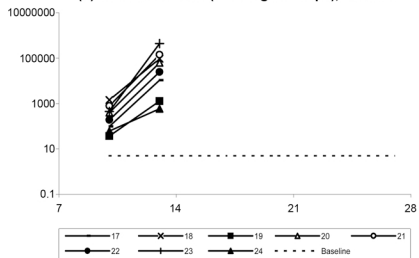
(b) 0.01 dose vaccine (challenged 21 dpv), Room 3



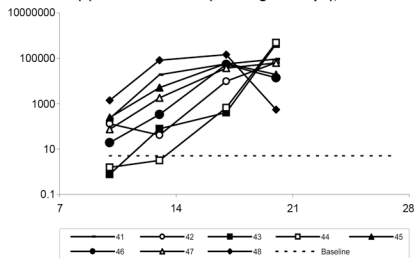
(c) 0.01 dose vaccine (challenged 28 dpv), Room 4



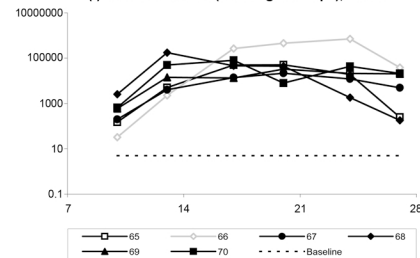
(d) 0.1 dose vaccine (challenged 14 dpv), Room 2



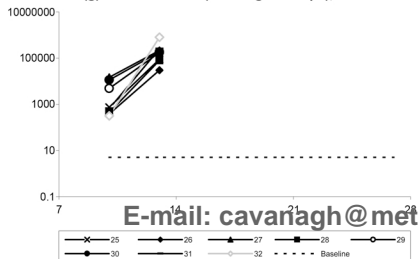
(e) 0.1 dose vaccine (challenged 21 dpv), Room 3



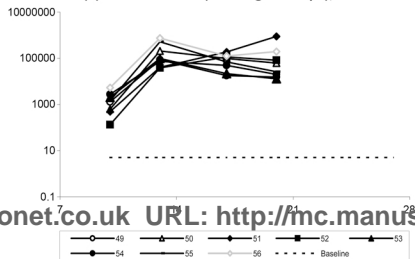
(f) 0.1 dose vaccine (challenged 28 dpv), Room 4



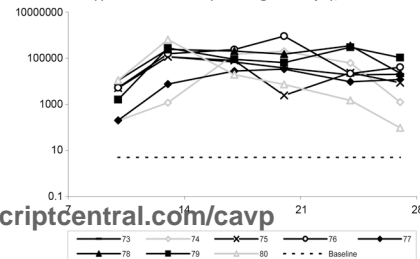
(g) 1 dose vaccine (challenged 14 dpv), Room 2



(h) 1 dose vaccine (challenged 21 dpv), Room 4

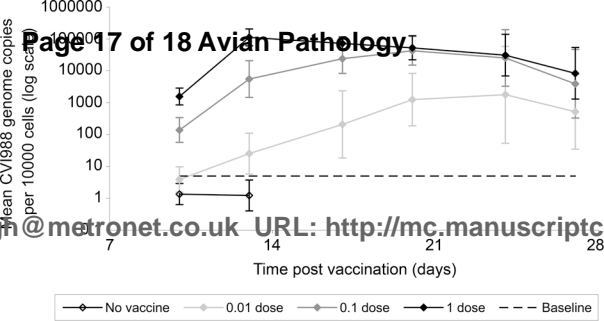


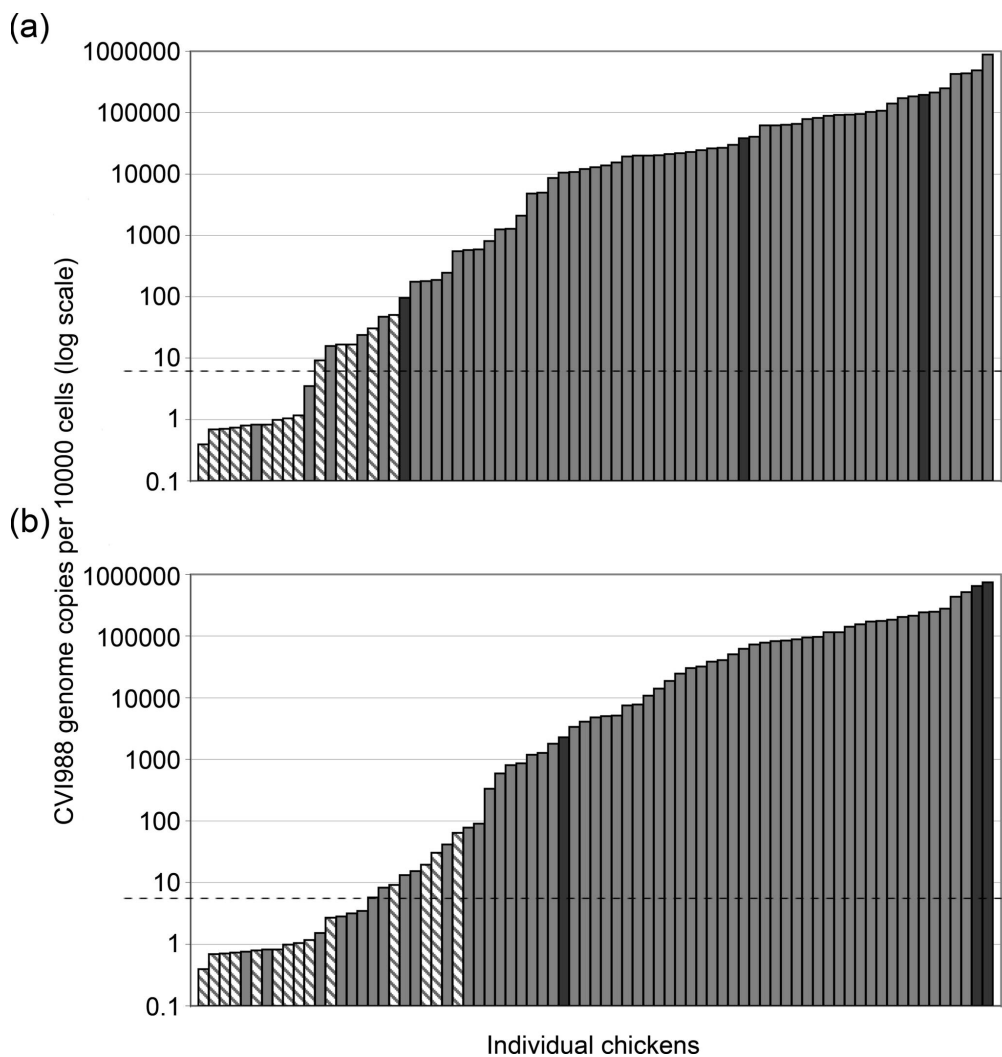
(i) 1 dose vaccine (challenged 28 dpv), Room 4



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Time post vaccination (days)





86x90mm (600 x 600 DPI)

